A STUDY OF THE NUTRITIONAL REQUIREMENTS AND TOXIN PRODUCTION OF CLOSTRIDIUM BOTULINUM TYPE F[1]

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Abstract

Clostridium botulinum type F was grown in a chemically defined medium containing 17 amino acids, 11 vitamins, glucose, and inorganic salts. The nutritional requirements were determined using single-omission test media. Arginine, tryptophan, tyrosine, valine, biotin, thiamin, and possibly methionine were essential nutrients. Growth was stimulated by glycine, isoleucine, phenylalanine, and para-aminobenzoic acid. Toxin was present in supernatant fluids from all synthetic medium cultures in which there was marked growth. In general, toxicity of synthetic medium cultures was about 1/10 that of complex medium cultures.

Toxin and precursor appear to be formed intracellularly, for both were released by rupturing young cells with sonic vibration. Protoxin could be activated by treatment with trypsin.

Introduction

The only known strain of *Clostridium botulinum* type F was isolated by Møller and Scheibel (7) from liver paste involved in an outbreak of human botulism on the Danish island of Langeland. This organism resembles, culturally, proteolytic strains of *C. botulinum* A and B, but the toxin that it produces is not identical with that of any of the other types of *C. botulinum* (2).

The present investigation was undertaken to determine the nutritional needs of *C. botulinum* type F as well as to investigate whether the toxin was produced intra- or extra-cellularly and whether it was synthesized as such or as a relatively non-toxic precursor that required activation before it became fully toxic. Several other types of *C. botulinum* seem to form part, at least, of their toxin as a non-toxic protoxin that must be activated by treatment with proteolytic enzymes before full toxicity is manifest (10).

Materials and Methods

Culture

The culture of *C. botulinum* type F was obtained from Dr. K. F. Meyer, G. W. Hooper Foundation, San Francisco, California.

Media

The complex medium contained 2.5 g trypticase,[2] 0.5 g yeast extract, 0.5 g glucose, 0.05 g sodium thioglycollate, and inorganic salts (50 mg KH_2PO_4,

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[2]Use of trade names is for identification only and does not constitute endorsement by the Public Health Service or the U.S. Department of Health, Education, and Welfare.

50 mg K_2HPO_4, 1 mg NaCl, 20 mg $MgSO_4$, 1 mg $FeSO_4$, and 0.82 mg $MnSO_4 \cdot H_2O$) in 100 ml distilled water. After the pH was adjusted to 7.4, 5-ml amounts were dispensed into 13 × 100 mm screw-cap tubes. The medium was sterilized at 121 °C for 15 minutes.

The chemically defined medium was composed of 17 amino acids, 11 vitamins, glucose, and inorganic salts. The constituents of the medium were identical with those used by Mager *et al.* (6), except that threonine, pyridoxal phosphate, and citrovorum factor were omitted and the inorganic salts were used in the same concentration as in the complex medium. Stock solutions of individual amino acids (except for cysteine hydrochloride) were sterilized at 121 °C for 15 minutes and stored at room temperature. Individual vitamin solutions were sterilized by Seitz filtration. Riboflavin and B_{12} were stored at room temperature in the dark; all other vitamin solutions were stored at 4 °C. Solutions of cysteine hydrochloride and sodium thioglycollate were not sterilized and were discarded after 2 weeks.

To prepare the chemically defined and the basal synthetic media, the appropriate amounts of the various stock solutions were mixed, the volume adjusted to 96 ml with distilled water, the pH adjusted to 7.4, and the medium filtered through a Seitz filter. It was then dispensed in 5-ml quantities into new, sterile, acid-cleaned 13 × 100 mm screw-cap tubes.

The basal synthetic medium was composed of arginine, glycine, isoleucine, leucine, methionine, phenylalanine, tryptophane, tyrosine, valine, biotin, para-aminobenzoic acid, thiamine, cysteine hydrochloride, glucose, sodium thioglycollate, and inorganic salts used in the same concentration as in the chemically defined medium.

Determination of Number of Cells in Cultures

The number of viable cells was determined by making 10-fold dilutions of cultures in physiological saline solution containing 0.05% sodium thioglycollate and using these dilutions to inoculate five replicate tubes of complex medium. The "most probable number" technique (Farber (4)) was used to calculate the results. Growth was also estimated from turbidity measurements made with a Coleman Jr. spectrophotometer, model 6 C, using light of 540 mμ wavelength.

Determination of Ability of Media to Support Growth from Small Inocula

This was determined by making "most probable number" determinations using a culture in synthetic medium for inoculum, diluting 10-fold in the physiological saline – thioglycollate diluent, and inoculating five replicate tubes of the medium being investigated. The reciprocal of the "most probable number" was taken as the smallest inoculum able to initiate growth.

Determination of Amino Acid and Vitamin Requirements

The stock culture of *C. botulinum* type F was transferred daily in the chemically defined medium to provide inoculum for the single-omission tests. The effect of such omissions was determined by measuring the turbidity of serial cultures in media from which one of the nutrients had been omitted. The possible growth-stimulating effect due to carryover of growth factors was eliminated by making three or more serial transfers. The initial inoculum in each set originated from a different daily stock culture transfer in complete

synthetic medium. The next serial transfer in the single-omission set and in the corresponding control medium were made from the previous transfer in single-omission medium. Serial transfers were made when maximal growth had been reached, in 18 to 24 hours when media from which a non-essential nutrient had been omitted were used, and at a later time when media from which a growth-stimulating nutrient had been omitted were used. The inocula were checked for ability to initiate growth at each transfer by inoculating a tube of complete synthetic medium. The amount of inoculum used was usually 0.25 ml per 5 ml of test medium.

Assay for Toxin

Toxin determinations were made with culture supernatant fluids diluted at half-log intervals in gelatin diluent (3). White mice, weighing 17 to 22 g, were inoculated intraperitoneally with 0.5 ml of each dilution and were observed at intervals for 4 days before being killed. The LD_{50} dose was calculated by the Tint and Gillen (9) modification of the Reed-Muench method.

Toxin Neutralization Tests

One part antitoxin was mixed with nine parts test material and the mixture incubated at 37 °C for 30 minutes. Nine parts test material and one part normal serum were treated similarly. White mice were inoculated with each mixture as described above.

Treatment with Sonic Vibration

Ten milliliters of culture fluid were pipetted into a 20-ml beaker that was surrounded by cracked ice. When the fluid had reached 6 °C, the probe of a Branson "Sonifier" was lowered into the beaker until it was about 1/2 in. from the bottom, the power supply was turned on and the current maintained at 6 A for 3 minutes. This treatment broke at least 90% of the cells, as determined by microscopic examination.

Results

Determination of Amino Acid and Vitamin Requirements

Results obtained in single-omission test media are presented in Tables I and II. The figures in these tables represent the average change in light transmission (percentage transmission immediately after inoculation less the percentage transmission when maximal growth was reached) of the third and all subsequent transfers in each set.

In nutritionally adequate media, maximal growth was reached in 18 to 24 hours. Although many cultures in synthetic medium appeared to lyse more rapidly than did those in complex medium, no marked lysis occurred in any cultures before 24 hours of incubation. In nutritionally adequate media, no increase in turbidity was apparent after incubation for 24 hours. In some single-omission test media, no increase in turbidity was apparent when cultures were incubated for longer periods. In other single-omission media, however, there was a continual increase in turbidity for several days. The nutrients that had been omitted from such media were considered growth stimulatory. When essential nutrients had been omitted, no increase in turbidity was apparent in the third serial transfer, even though the inoculum contained enough viable cells to induce growth in the control medium.

TABLE I

Growth of *C. botulinum* type F in amino acid test media

	Change in % transmission			
	Single-omission test media			Complete synthetic control media
Amino acid omitted	24 h	48 h	72 h	24 h
Non-essential				
Alanine	51*			59
Aspartic acid	46			49
Cysteine	50			62
Glutamic acid	49			44
Histidine	36			44
Leucine	31			39
Lysine	41			47
Proline	47			47
Serine	45			48
Stimulatory				
Glycine	10	14	26	45
Isoleucine	14	18		39
Phenylalanine	12	16		50
Essential				
Arginine	−4			44
Tryptophan (L, DL)	−1			31
Tyrosine	2			34
Valine	−3			47

*Figures represent the average of all third and subsequent transfers.

TABLE II

Growth of *C. botulinum* type F in vitamin test media

	Change in % transmission	
Vitamin omitted	Single-omission test media, 24 h	Complete synthetic control media, 24 h
Non-essential		
B_{12}	47*	46
Choline	58	52
Calcium pantothenate	48	59
Folic acid	58	52
Inositol	55	59
Nicotinic acid, nicotinamide	49	59
Pyridoxal, pyridoxine, pyridoxamine	43	45
Riboflavin	49	60
Stimulatory		
Para-aminobenzoic acid	8†	64
Essential		
Biotin	0	39
Thiamin	1	43

*Figures represent the average of all third and subsequent transfers.
†Change in % transmission = 38 units after 96 hours.

The data in Tables I and II show that arginine, tryptophane, tyrosine, valine, biotin, and thiamine were essential for growth. Glycine, isoleucine, phenylalanine, and para-aminobenzoic acid, although not essential, greatly stimulated growth.

Experiments using methionine-deficient media gave equivocal results. In four series of tests, growth was slow and slight in the first several transfers. In some of these, there was no growth after the fourth or fifth transfer. From these results, methionine appears to be an essential nutrient. Nevertheless, in other experiments, the cultures in methionine-deficient medium grew slowly and sparsely for five or six serial transfers, then more rapidly and heavily in subsequent transfers. We interpreted this as indicating that we had selected a methionine-independent mutant. However, no evidence to support this interpretation could be obtained. Consequently, it seems that methionine should be considered as essential nutrient for *C. botulinum* type F.

Growth in a basal synthetic medium composed of essential and stimulatory amino acids and vitamins was somewhat slow and sparse, the average change in transmission being 36% compared to 58% for the complete synthetic medium. Growth was not improved when the concentration of each nutrient was doubled, or when the other nutrients present in the complete medium were individually added to the basal medium.

Growth in Complete Synthetic Medium Compared with Growth in Complex Medium

Determination of the numbers of cells in fully grown cultures.—Growth in synthetic medium compared favorably with growth in complex medium. At the end of the log phase of growth, cultures in synthetic and complex media were found to contain 4.9×10^8 cells per milliliter each. This observation was confirmed in two similar experiments.

Determination of ability of media to initiate growth from small inocula.—From the reciprocal of the "most probable number", it was found that about 5×10^{-6} ml of culture was required to initiate growth in complex medium, and about 6×10^{-6} of the same culture was required to initiate growth in the synthetic medium.

Toxicity of Cultures in Synthetic and Complex Media

Toxin could be demonstrated in culture supernatant fluids from all media in which there was marked growth; i.e., in media containing all essential and stimulatory nutrients. In general, the toxicity of supernatant fluids from cultures in single-omission media was comparable to that of cultures in complete synthetic medium and ranged from 450 to 6300 mouse intraperitoneal LD_{50} doses per milliliter. Supernatant fluids from cultures in synthetic medium were usually about 1/10 as toxic as those from complex medium.

Colony Types

Four distinctly different colony types were recognized and separated: TOX (mosaic pattern); TP (translucent, flat, spreading); OPY (opaque, patterned, yellowish); and OG (opaque, granular). Several intermediate forms were also observed. The TOX and TP phases appeared to be identical with those described by Dolman and Murakami (2). The OG colonies were bluish (OGB) or yellowish (OGY). The yellow colonies (OGY and OPY) contained many sporulating rods, whereas few, if any, sporulating rods were seen in smears made from colonies of the other types.

Although cultures of all colony types mutated to other colonial forms, the mutation rate varied from one phase to another. Cultures of TOX and TP

colonies were the most stable and cultures of OGY were the least stable. In fact, some OGB colonies were present on all plates made from broth cultures grown from well-isolated OGY colonies. Many TOX and TP phase cultures remained stable for 4 to 8 weeks when transferred daily and for as long as 6 months when frozen in brain medium.

When the different colonial phases had been separated, they were subcultured in complex and in synthetic media for at least three serial transfers, and supernatant fluids from 3-day cultures were titrated for toxicity in mice. The results are presented in Table III. Some cultures produced relatively small amounts of toxin, particularly when grown in the synthetic medium.

TABLE III

Toxicities of 3-day culture supernatant fluids from different colony types in complex and synthetic media

Colony type* and identification	Mouse IP LD_{50} per ml		Toxicity ratio, complex : synthetic
	Complex	Synthetic	
TOX-27	20,000	2000	10
TP-04	10,000	1000	10
INT-32	80	90	0.9
OGB-30	200	35	6
TP-4a	4200	<64	>66
OGY-31	3000	<20	>150
OGB-34	6400	20	320
OPY-26	10,500	14	720

*TOX = mosaic pattern; TP = translucent, flat; INT = intermediate (slightly patterned); OGB = opaque, granular, bluish; OGY = opaque, granular, yellowish; OPY = opaque, patterned, yellowish.

Production of Toxin and Precursor

Cultures OGB-34 and OPY-26, having the highest complex–synthetic medium toxicity ratios, were selected for preliminary studies. Tubes containing 5 ml of medium were inoculated near the bottom with an actively growing culture and incubated at 30 °C for 16 to 22 hours. The contents of replicate tubes were pooled and divided into 5-, 10-, and 15-ml samples. The 5-ml sample was incubated for an additional 2 days to determine the toxicity that would be attained during the usual incubation period. The 10-ml samples and the supernatant fluids from the 15-ml samples were treated with sonic vibration and with crude trypsin. The amount of toxin precursor was assumed to correspond to the increased toxicity after treatment with trypsin. The amount of intracellular toxin or precursor was assumed to correspond to that released by sonic treatment.

The results presented in Table IV (experiment 1) show that toxicity was increased when the whole culture was treated with sonic vibration. Intracellular precursor was demonstrated in all four cultures, for toxicity was increased 2 to 10 times when the whole culture, after treatment with sonic vibration, was also treated with trypsin.

In two instances, OPY-26 in synthetic medium and OGB-34 in complex medium, the supernatant fluid from the young cultures contained detectable protoxin, for the untreated fluid was not demonstrably toxic for mice, whereas

TABLE IV

Toxicity of cultures of *C. botulinum* type F on complex and synthetic medium after treatment with sonic vibration and with trypsin

Material tested	Culture age (h)	Treatment*	Mouse IP LD_{50} per ml			
			OPY-26		OGB-34	
			Complex	Synthetic	Complex	Synthetic
Experiment 1						
Supernatant fluid	16–22	—	<10	<10	<10	<10
		T	<100	64	64	<10
		S	<10	<10	<10	<10
Whole culture	16–22	S	900	64	640	43
		ST	2000	640	4800	640
Supernatant fluid	68	—	9400	10	6400	<10
		T	2000	640	2000	20
		S	9000	10	6400	<10
Experiment 2						
Supernatant fluid	20	—				10
		T				10
Whole culture	20	—				64
		T				2000
Supernatant fluid	68	—				10
		T				10
Whole culture	68	—				64
		T				1000
Experiment 3						
Supernatant fluid	20	—		<10		
		T		64		
Whole culture	20	S_o		64		
		ST		640		
Supernatant fluid from S_o		—		64		
		T		640		
Resuspended sediment from S_o		—		<10		
		T		<10		

*— = no treatment; S = treatment with sonic vibration; T = treatment with trypsin.

the trypsin-treated fluid was so. It seems likely that the precursor had leaked through the cell wall or that lysis of a small proportion of the cells had taken place. However, no cellular debris was seen in Gram-stained preparations of any of the young cultures. The small amount of toxin in the supernatant fluid from the 3-day culture of OGB-34 in synthetic medium indicated that intracellular toxin or precursor might still be present in a culture of this age. Accordingly, supernatant fluid and whole cultures treated with sonic vibration were tested for toxin and protoxin after growth for 20 and 68 hours. The data in Table IV (experiment 2) indicate that both intracellular toxin and precursor were present in 3-day-old cultures.

The data from these experiments indicate that the toxin and the precursor are released into the medium by sonic vibration. There is no evidence, however, that the toxin and precursor were truly endocellular in origin or whether they were associated with cell wall fragments. To obtain information on this point, a young culture of OPY-26 in synthetic medium was treated with sonic vibration and centrifuged at 10,000 *G* for 15 minutes. Samples of supernatant fluids, culture treated with sonic vibration, and the supernatant fluid and resuspended sediment from the latter were tested for toxicity in mice before

TABLE V

Toxicity of cultures of *C. botulinum* type F after treatment with sonic vibration and trypsin

Culture	Log phase culture				Three-day culture			
	Supernatant fluid		Whole culture + SV*		Supernatant fluid		Whole culture + SV	
	No trypsin	With trypsin	No trypsin	With trypsin	No trypsin	With trypsin	No trypsin	With trypsin
TOX-01	<10†	20	2,000	6,400	64,000	64,000	NT‡	NT
TOX-60	20	40	640	2,000	64,000	64,000	64,000	64,000
TOX-67	<10	<10	<10	20	<10	20	<10	64
TP-04	<10	<10	640	6,400	≧6,400	9,400	NT	NT
TP-68	10	20	64	64	640	200	640	640
OGB-37	<10	<10	<10	64	100	100	NT	NT
OGB-69	200	64	2,000	20,000	64,000	64,000	64,000	64,000
OGY-64	64	64	640	2,000	6,400	640	6,400	2,000

* = Treated with sonic vibration.
† = Mouse IP LD_{50} per milliliter.
‡ = Not tested.

and after treatment with trypsin. The results, presented in Table IV (experiment 3), indicate that neither toxin nor precursor was associated with sedimentable fractions, i.e., residual intact cells or cell wall fragments, but remained as soluble components in the supernatant fluid.

The previous experiments had demonstrated that toxin was formed intracellularly and that it may have been formed as a precursor or protoxin. However, the strains selected for these experiments had been chosen because of their markedly different production of toxin when grown in complex and in synthetic media. Therefore, similar experiments were conducted with cultures of other colony types grown in complex medium. Representative results, presented in Table V, show that toxin was produced intracellularly in cultures of all colony types. Toxin precursor was demonstrated in some cultures and not in others. TOX cultures were among both the most and the least toxic of the cultures tested. Toxin of all these cultures was found to be neutralized by *C. botulinum* type F antitoxin.

The results of the preceding experiments show that both intracellular toxin and intracellular precursor may be found in the same culture. The cultures tested, however, might represent either a population of individual cells containing both toxin and precursor, or a mixture of older organisms in which the precursor had been activated and younger organisms in which it had not. To obtain more information on the possibility of intracellular toxin and intracellular precursor being found in the same bacterial cells, TOX-60 was transferred every 12 h in complex medium for three transfers and used as inoculum for a flask of medium which was incubated in a Brewer jar while the contents of the flask were being continuously mixed by means of a magnetic stirring bar. Samples were removed at 16, 40, and 64 hours incubation and tested for intracellular toxin and precursor as described above. The results of this experiment, presented in Table VI, indicate that both toxin and precursor were present at the same time within the cells of this culture and that either intracellular activation of precursor or the intracellular synthesis of fully active toxin took place.

TABLE VI

Toxicity of stirred TOX-60 culture after treatment with sonic vibration and trypsin

Material tested	Culture age (h)	Treatment*	Mouse IP LD_{50} per ml
Supernatant fluid	16	—	64
		T	92
Whole culture	16	S	920
		ST	6,400
Supernatant fluid	40	—	44,000
		T	20,000
Whole culture	40	S	64,000
		ST	14,000
Supernatant fluid	64	—	920
		T	440
Whole culture	64	S	6,400
		ST	6,400

* — = no treatment; T = treatment with trypsin; S = treatment with sonic vibration; ST = treatment with sonic vibration and trypsin.

Discussion

C. botulinum type F, like proteolytic strains of types A and B, can be cultivated in a chemically defined medium. When examined at the end of the log phase of growth, cultures in synthetic medium contained as many viable organisms as did cultures in complex medium, as measured by the "most probable number" technique. Toxin production in synthetic medium was considerably less than that in complex medium. It is apparent that toxin production is a function of factors other than the total number of viable organisms present in a culture at the end of the log phase of growth. Similar conclusions were arrived at by Mager *et al.* (6) regarding the toxicity of type A strains.

The amino acid and vitamin requirements of type F are similar to those of proteolytic strains of types A and B: arginine, tryptophane, tyrosine, valine, biotin, and thiamine are essential; glycine, isoleucine, phenylalanine, and para-aminobenzoic acid are stimulatory. Methionine may be essential for growth; it certainly is essential for optimal growth. Roessler and Brewer (8), Mager *et al.* (6), and Kindler *et al.* (5) found that essentially these same amino acids were necessary to obtain good growth of type A strains. Glycine, stimulatory for type F, apparently is not required by type A strains, while threonine, necessary for type A, is not necessary for type F.

The requirement for methionine seems definite but variable. It may depend, to a considerable extent, on a delicate balance among growth factors, particularly between cysteine and methionine. Mager *et al.* (6) found that methionine could be partially replaced for type A strains by cysteine.

The results obtained in this study indicate that type F toxin and protoxin are formed intracellularly, for both were released by rupturing young cells with sonic vibration. There was no evidence that either the toxin or its precursor was closely bound to fragments of the cells, for centrifugation at 10,000 *g* did not cause any decrease in toxic activity of a sonic-treated preparation. Apparently, the toxin and its precursor are "protoplasmic" rather than cell wall toxins or true exotoxins. The relative proportions of precursor : toxin in different cultures varied considerably. With young cultures, as much as 90% of the potential toxin apparently existed in the form of the precursor. With older cultures, the greater portion of the precursor usually had been converted to toxin.

Although treatment with trypsin serves to convert precursor to toxin, the toxin itself seems to be somewhat susceptible to the further action of this enzyme. Data presented in Tables IV, V, and VI show that toxic activity can be decreased in some specimens by the same treatment with trypsin that markedly increased it in others. Decreases in toxicity to 1/10 of the original value were not uncommon. Therefore, the apparent "activation" or "inactivation" brought about by treatment with trypsin should be considered to be the difference between the amount of precursor truly activated by the trypsin and the amount of toxin that had been truly inactivated by the same process. In those instances where the amount of precursor rendered toxic by trypsin treatment exceeded the amount of toxin rendered non-toxic, apparent activation occurred. Where the amount of toxin rendered non-toxic

exceeded the amount of precursor rendered toxic, apparent inactivation occurred.

Colonial morphological characteristics and the formation of toxin or precursor do not appear to be closely related in type F cultures, as seems to be the case with strains of type E (1). Some cultures of all the different colony types that we examined were found to produce precursor or toxin. Apparently, toxin production in this type F strain is an extremely stable characteristic.

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